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Monitoring Biolog patterns and *r/K*-strategists in the intensive culture of *Artemia* juveniles

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L. VERSCHUERE, J. DHONT, P. SORGELOOS AND W. VERSTRAETE. 1997. *Artemia* juveniles were cultured under intensive conditions in three series of six tanks at different times. Both plate counts and Biolog GN microtitre plates were used to monitor the microbial community. Repetitions of the *Artemia* cultures in time revealed significant differences in Biolog patterns and bacterial counts, showing that normal culture practices, including chlorination of the sea water, does not allow control of the associated microbial community. However, the similarity among the Biolog fingerprints of all 18 tanks as determined by the Pearson correlation coefficient was always high. Both observations together indicate that the microbial community which colonizes the culture tanks seems to be determined by both deterministic factors (e.g. salinity, temperature, oxygen concentration, composition of the feed) and stochastic factors (micro-organisms still present in the sea water after chlorination, on the walls of the culture tanks or in the air around the culture tanks). When a high proportion of microbial *r*-strategists was present in the water at the beginning of the *Artemia* culture, the smallest differences in Biolog patterns among the tanks of one series throughout the culture period were observed. A parallelism between *Artemia* rearing success, functional fingerprint of the bacterial community and the proportion of r-strategists present, was observed. This suggests an important role of the bacterial community in the overall *Artemia* rearing success.

INTRODUCTION

In aquaculture, and especially in larviculture, microbiology has become increasingly important as production systems have become more intensive and sophisticated. Gaining control over microbiology to improve production becomes essential for the economic viability of modern aquaculture ventures. Initially, microbiological research in aquaculture focused on the understanding of deleterious effects caused by pathogens, and on the use of chemicals or antibiotics to obtain quantitative control of bacteria. Recently, alternative approaches have been proposed, taking into consideration some principles of microbial ecology and current knowledge of the interactions between bacteria and cultured organisms. Successful application of probionts in crustacean (Nogami and Maeda 1992) and marine fish larviculture (Gatesoupe 1991; Strøm and Ringø 1993), and the results obtained with microbial matured water in turbot *Scophthalmus maximus* larviculture (Vadstein *et al.* 1993; Salvesen *et al.* 1995), illustrate the importance of gaining control of bacterial communities and the possibilities offered by these alternative approaches.

Intensive rearing of aquaculture species and their live prey often requires that feed is applied at a high concentration. This results in an excellent medium for the growth of heterotrophic bacteria, which might include opportunistic pathogens. The feed, which is often composed of substances not abounding in the natural aquatic environment, can be used as substrate by bacteria which in nature would have restricted opportunities to grow.

The intensive culture of *Artemia* as a live prey for fish and shrimp larvae has always suffered from unpredictable results due to incidental crashes in individual production tanks. Several authors reported on the influence of bacteria on *Artemia* (D'Agostino and Provasoli 1968; Solangi *et al.* 1979; Gunther and Catena 1980; Douillet 1987; Intriago and Jones 1993; Puente *et al.* 1992; Rosowski *et al.* 1992), but no data were

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found on the exact relationship between the quantitative or qualitative composition of the microbial community and the rearing success of *Artemia* under open and intensive culture conditions. As, generally, no microbial control is performed except chlorination of the sea water (Sorgeloos *et al.* 1986) or sporadic use of antibiotics (Rosowski *et al.* 1992), the composition of the microbial community is left to chance and may therefore be highly variable in time and between culture tanks. Furthermore, several bacterial species have been reported to be a source of infections, causing high mortalities among fish larvae. Live prey such as the rotifer, *Brachionus plicatilis*, and *Artemia* are often suspected of being vectors of these bacterial infections (Muroga *et al.* 1987; Nicolas *et al.* 1989; Perez-Benavente and Gatesoupe 1988; Tanasomwang and Muroga 1990).

The Biolog redox technology (Biolog Inc., Hayward, CA, USA), based on a tetrazolium dye reduction as an indicator for sole carbon source utilization, can be used as a rapid community level method to characterize and classify heterotrophic microbial communities. It is currently used on several types of microbial communities and habitats such as soil (Garland and Mills, 1991; Zak *et al.* 1994; Haack *et al.* 1995; Wünsche *et al.* 1995), water (Garland and Mills 1991), plant-associated microbial communities (Garland and Mills 1994; Ellis *et al.* 1995; Heuer *et al.* 1995), activated sludge (Victorio *et al.* 1996), rhizosphere samples (Garland and Mills 1991; Garland, 1996a, b) and composted manure (Insam *et al.* 1996).

This study confirms that the Biolog system can be used to monitor the microbial community of intensive *Artemia* rearing. It is argued that it could be a useful tool for assessing the control of the microbial community, helping to increase the predictability of intensive *Artemia* cultures.

MATERIALS AND METHODS

Zootechnical aspects

The culture of *Artemia* was undertaken in six rectangular 100 l polyethylene tanks. Aeration was insured by three perforated PVC tubes per tank. The air flow per tank was approximately 15 l min^{-1}. The aeration air originated from the open air and was filtered through autoclaved cotton. The tanks were filled with natural sea water from the Belgian coast (North Sea) which was filtered over a 10 μm filter bag and disinfected in the tanks with 1 mg l^{-1} NaOCl. This disinfection phase lasted for 1 h, during which no aeration was provided. Residual chlorine was then removed by strong aeration for 8 h. The sea water was then ready to use.

The temperature of the culture water was maintained between 25 and 28°C. Feed consisted of a micronized 80/20% mixture of pea and corn residue which was kept at 4°C. It was distributed semi-continuously as a suspension using a timer-controlled peristaltic pump. Optimal feed levels, maintained using a standard feeding regime, consisted of 20, 22·5, 25, ? 32·5 and 35 g/100 l^{-1} for day one, two, three, four, five and six, respectively.

Artemia cysts (60 g; EG-grade, INVE Aquaculture NV, Baasrode, Belgium) were hatched and the nauplii harvested following standard methods described by Sorgeloos *et al.* (1986). When the nauplii were put in the 100 l tanks, initial animal density was about 10 ml^{-1}. From then on, the culture lasted 6 d, after which biomass was harvested and frozen.

Animal density and body length were measured after 12, 84 and 132 h. Animal density was determined by counting four samples of 10 ml, fixed and stained with lugol, under a binocular microscope. Survival was expressed as a percentage of living animals compared to the animal density at 12 h. This time span of 12 h was necessary to allow the remaining cysts to hatch. Body length was measured using a microscope equipped with a drawing mirror and calculated using a digitizer. Biomass calculation was based on animal density and body length rather than weight, because important weighing errors caused by flocs and other debris in the culture water could not be avoided. Individual dry weight (IDW) and biomass concentration (BC) were calculated following the formula proposed by Abreu-Grobois *et al.* (1991):

$$\mathrm{IDW} = 10^{[-2{\cdot}53 + 1{\cdot}63 \times \log(\mathrm{length}) + 0{\cdot}81 \times (\log(\mathrm{length}))^2]} \times 1000\ (\mu g)$$

$$\mathrm{BC} = \frac{1}{1000} \times \mathrm{IDW} \times \text{number of animals/l}\ (\mathrm{mg\ l^{-1}})$$

The zootechnical data were statistically analysed through ANOVA and the Duncan test to detect significant differences.

At three subsequent times, six tanks were set up under identical conditions as described above. The first series of six tanks was set up on 15 May (T1–6), the second on 4 June (T7–12) and the third on 24 June 1995 (T13–18). From the zootechnical point of view, they may be considered as replicates.

Plate counts

The culture water was filtered over a 100 μm screen, serially diluted in autoclaved nine salt solution (NSS, Olsson *et al.* 1992) and plated out on marine agar 2216 (Difco Laboratories, Detroit, MI, USA) for a total count of marine heterotrophic bacteria, TCBS cholera medium (Oxoid, Unipath Ltd, Basingstoke, UK) for *Vibrio* spp., and *Pseudomonas* agar base with *Pseudomonas* C-F-C-supplement (Oxoid, Unipath Ltd, Basingstoke, UK) for *Pseudomonas* spp. The plates were incubated at 28°C and the colony counts were registered after 2 d for TCBS and *Pseudomonas* agar and 5 d for marine agar. For the determination of the amount of bacteria occurring on the surface and in the gut of the *Artemia*, 20 ml of the culture water containing the *Artemia* were filtered over a 100 μm filter under sterile conditions. The *Artemia* on the filter were

washed twice using 10 ml of sterile NSS. Subsequently, the filter with the organisms and 20 ml of sterile NSS were transferred to a sterile plastic bag and treated for 5 min in a stomacher blender (400SN, Seward Medical; London, UK) in order to dislodge surface and intestinal bacteria. This suspension was also serially diluted and plated as described above. The results of the plate counts were statistically analyzed after log-transformation, through ANOVA and the Duncan test.

The inputs of bacteria in the system were evaluated through plate counts on marine agar. They were performed on ready to use chlorinated sea water and feed (freshly prepared and after 24 h at 4°C). The aeration and ambient air was sampled according to the gas-scrubbing flask method (Verstraete and Voets 1974).

r- and *K*-strategists

An agar plating method based on the findings of De Leij *et al.* (1993) provided an ecological fingerprint of microbial communities. This method is based on the quantification of the numbers of colonies that have appeared on the marine agar plates after 2 and 5 d of incubation at 28°C. Fast-growing bacteria yielding visible colonies within 2 d of incubation were considered *r*-strategists, while those yielding visible colonies between 2 and 5 d were considered *K*-strategists. After 5 d of incubation, all the colony forming units (cfu) are assumed to be visible on the plates and the fraction of *r*- or *K*-strategists can be calculated and expressed as percentages. There is actually no clear border between r- and K-strategists, but a continuous transition exists between the two types (Andrews and Harris 1986). The time limits were chosen arbitrarily, but seemed to be a reasonable division of the experimental incubation time for the agar plates. Significant differences in percentage of *r*-strategists between sampling times and tank series were determined with one-way ANOVA and the Duncan test on Arcsinus-transformed data.

Microbial community analysis using Biolog

The GN microtitre plates. Commercially available, 96-well, Gram-negative (GN) microtitre plates (Biolog Inc.) were used containing 95 different carbon sources and a control well without carbon source. Each well also contained nutrients, salts, a small amount of peptone and the redox dye, tetrazolium violet. If the bacteria are able to oxidize the carbon source, the dye is reduced during respiratory activity and insoluble formazan (violet) accumulates inside the cells (Bochner and Savageau 1977).

Inoculum density. Preliminary tests showed the necessity to dilute the culture water because of the abundance of organic matter in it and, consequently, intensive colouring in the control well. The microbial biomass present in the culture water was measured as microbial ATP using the Biocounter 2500 (Lumac bv; Landgraaf, The Netherlands). ATP measurements are based on the light-generating reaction of the nucleotide with luciferin and firefly luciferase (Van de Werf *et al.* 1985). A volume of 100 μl of the 100 μm-filtered culture water was put in a 5 ml glass vial and inserted into the Biocounter. A volume of 100 μl of the microbial ATP-releasing agent (NRB, a quaternary detergent, Lumac bv) was automatically added and allowed to react during 10 s. Then, a volume of 100 μl of the luciferase enzyme preparation (Lumac bv) was injected into the vial and the integrated light emission was measured over a period of 10 s. Two luminescence measurements were performed for each water sample. To convert the relative light units to ATP concentration, a standard has been prepared for each luciferase enzyme preparation used. In order to obtain a similar inoculum density, the samples were diluted with sterile NSS to an ATP-content of 5 pg ml^{-1}, corresponding to 10^5–10^6 cells ml^{-1}.

Data collection. The diluted culture water samples with equal ATP content were inoculated into the plates with a multipipette and incubated at 28°C. The optical density (O.D.) produced from the reduction of tetrazolium violet in each well was measured at 590 nm using a bio-kinetics reader EL312e and the KinetiCalc EIA application software release 2.03 (Bio-tek Instruments Inc., Winooski, VT, USA) and corrected for the value in the control well. The O.D. development was measured after 24, 30, 36 and 48 h of incubation. Unless otherwise specified, results are given after 24 h of incubation.

Functional diversity. The functional diversity of the analysed microbial community can be quantified using a substrate diversity index (Zak *et al.* 1994):

$$H = -\Sigma[p_i \ln (p_i)]$$

where p_i is the ratio of the corrected $O.D._{\cdot 590}$ on a carbon source (i) to the sum of the corrected $O.D._{\cdot 590}$ on all substrates. The substrate equitability (J) is a rescaling of the substrate diversity:

$$J = H/H_{max}$$

with H_{max} the maximal substrate diversity index for the plates, assuming that each of the 95 carbon sources would cause the same colour intensity. A J value close to 1 indicates that the bacteria grow in the same proportion on each of the carbon sources, while a value close to 0 suggests that only a very few carbon sources produce an intensive colour of the well.

Multivariate analysis. The relationship in Biolog pattern among the different culture tanks and sampling times was determined by multivariate statistical techniques, such as cluster analysis (CA) and principal component analysis (PCA), after subtraction of the control. CA was performed on the corrected $O.D._{590}$ for each carbon source as variable, using the squared Euclidian distance as a similarity index and the average linkage method between groups to cluster the cases. The results of the CA are shown on a relative scale in a dendrogram. PCA was performed on the corrected $O.D._{590}$ for each carbon source as variable with three principal components and a varimax rotation.

Pearson correlation coefficient. To quantify the similarity between the sole carbon utilization patterns of microbial community A and B, the Pearson correlation coefficient was calculated as follows (Randerson 1993):

$$r_{A,B} = \frac{covariance_{A,B}}{\sqrt{variance_A \times variance_B}}$$

Statistical analysis

Statistical analysis was performed with the software SPSS release 6.0 (SPSS Belgium, Heuerlee, Belgium) and at a significance level of 0·05.

Sampling scheme

Day −1: hatching of the cysts.
Day 0: plate counts of the disinfected culture water; the nauplii were separated from the shells, rinsed, and put in the 100 l culture tanks.
Day 1, after 12 h: sample of the culture water for Biolog, animal density.
Day 2, after 36 h: culture water for Biolog and plate counts on water + *Artemia*.
Day 4, after 84 h: culture water for Biolog and plate counts on water + *Artemia*, animal density and body length.
Day 6, after 132 h: culture water for Biolog and plate counts on water + *Artemia*, animal density and body length.

RESULTS

Plate counts

Before the nauplii were put into the 100 l culture tanks, the chlorinated sea water was plated out on marine agar to assess the efficiency of the disinfection. This gave densities ranging from 1·9 to $7{\cdot}1 \times 10^3$ cfu ml^{-1} for tanks one to six. The feed also appeared to contribute to the initial colonization of the chlorinated sea water, as freshly prepared feed yielded $2{\cdot}3 \times 10^6$ cfu g^{-1} on marine agar, while after 24 h of conservation of the feed at 4°C, the total density of marine heterotrophic bacteria was $8{\cdot}1 \times 10^6$ cfu g^{-1}. Considering the daily feed weight and the volume of the culture tanks, the bacterial input through the feed amounted to $\pm 10^3$ cfu ml^{-1} d^{-1}. The bacterial input through aeration was found to be negligible (± 50 cfu l^{-1} d^{-1}).

The bacterial counts of the culture water as a function of the sampling time are given in Table 1. A very strong and rapid increase in the total number of culturable marine heterotrophic bacteria up to 10^8–10^9 cfu ml^{-1} at the beginning of the culture period, and a subsequent decrease with 2 log-units, was observed. Significant differences between the three tank series were found in the bacterial numbers detected on marine agar, TCBS and *Pseudomonas* agar, indicating that differences exist in the microbial community of the culture waters. The density of *Vibrio* spp. in the culture water and, to a lesser extent, of *Pseudomonas* spp., was the highest in the tanks of series three for the three sampling times. Generally, the proportion of *Vibrio* spp. and *Pseudomonas* spp. in the culture water compared to the plate counts on marine agar increases with the sampling time.

The changes in the densities of the *Artemia*-associated bacteria as a function of time are less drastic than those in the culture water (Table 2). An increase with sampling time in the numbers of gut- and surface-associated bacteria is noticeable on marine agar, TCBS and *Pseudomonas* agar with 0·75–1·35, 0·92–2·12 and 1·0–2·67 log-units, respectively. On the three agar types, significant differences were found between the three tank series, again indicating different microbial communities developed on the *Artemia*. No clear

Table 1 Log-values of the plate counts on marine agar (MA), TCBS cholera medium (TCBS) and *Pseudomonas* agar (PA) of the culture water of the three tank series (mean ± S.D., in log cfu ml^{-1}). Initial densities amounted to 10^3–10^4 cfu ml^{-1}

		Sampling time (h)		
	Series	36	84	132
MA	T1–6	$8{\cdot}57 \pm 0{\cdot}15^{a*}$	$7{\cdot}68 \pm 0{\cdot}25^{a}$	$6{\cdot}89 \pm 0{\cdot}27^{a}$
	T7–12	$8{\cdot}48 \pm 0{\cdot}19^{a}$	$7{\cdot}20 \pm 0{\cdot}30^{b}$	$6{\cdot}16 \pm 0{\cdot}61^{b}$
	T13–18	$8{\cdot}68 \pm 0{\cdot}12^{a}$	$7{\cdot}56 \pm 0{\cdot}30^{a}$	$6{\cdot}73 \pm 0{\cdot}22^{a}$
TCBS	T1–6	$5{\cdot}73 \pm 0{\cdot}27^{a}$	$4{\cdot}99 \pm 0{\cdot}52^{a}$	$4{\cdot}30 \pm 0{\cdot}30^{a}$
	T7–12	$6{\cdot}41 \pm 0{\cdot}36^{b}$	$4{\cdot}67 \pm 0{\cdot}30^{a}$	$4{\cdot}73 \pm 0{\cdot}12^{a}$
	T13–18	$6{\cdot}69 \pm 0{\cdot}36^{b}$	$5{\cdot}46 \pm 0{\cdot}30^{b}$	$5{\cdot}74 \pm 0{\cdot}79^{b}$
PA	T1–6	$4{\cdot}63 \pm 0{\cdot}23^{a}$	$3{\cdot}94 \pm 0{\cdot}58^{a}$	$3{\cdot}39 \pm 0{\cdot}43^{a}$
	T7–12	$4{\cdot}27 \pm 0{\cdot}98^{ab}$	$5{\cdot}20 \pm 0{\cdot}26^{b}$	$4{\cdot}44 \pm 1{\cdot}86^{ab}$
	T13–18	$5{\cdot}47 \pm 0{\cdot}71^{b}$	$5{\cdot}16 \pm 0{\cdot}31^{b}$	$4{\cdot}87 \pm 0{\cdot}47^{b}$

Figures with the same letter are not significantly different from each other for a particular agar type and sampling time ($\alpha = 0{\cdot}05$).

Table 2 Log-values of the plate counts on marine agar (MA), TCBS cholera medium (TCBS) and *Pseudomonas* agar (PA) of the *Artemia*-associated bacteria of the three tank series (mean ± S.D., in log cfu *Artemia*$^{-1}$)

		Sampling time (h)		
	Series	36	84	132
MA	T1–6	$5{\cdot}99 \pm 0{\cdot}45^{a*}$	$6{\cdot}11 \pm 0{\cdot}21^{a}$	$6{\cdot}73 \pm 0{\cdot}42^{a}$
	T7–12	$5{\cdot}23 \pm 0{\cdot}42^{b}$	$6{\cdot}35 \pm 0{\cdot}50^{ab}$	$6{\cdot}23 \pm 0{\cdot}33^{b}$
	T13–18	$5{\cdot}27 \pm 0{\cdot}30^{b}$	$6{\cdot}57 \pm 0{\cdot}25^{b}$	$6{\cdot}62 \pm 0{\cdot}18^{ab}$
TCBS	T1–6	$3{\cdot}85 \pm 0{\cdot}56^{a}$	$3{\cdot}50 \pm 0{\cdot}34^{a}$	$4{\cdot}42 \pm 0{\cdot}14^{a}$
	T7–12	$3{\cdot}02 \pm 0{\cdot}43^{b}$	$4{\cdot}60 \pm 0{\cdot}27^{b}$	$5{\cdot}14 \pm 0{\cdot}41^{b}$
	T13–18	$3{\cdot}70 \pm 0{\cdot}37^{a}$	$4{\cdot}71 \pm 0{\cdot}51^{b}$	$5{\cdot}29 \pm 0{\cdot}31^{b}$
PA	T1–6	$3{\cdot}34 \pm 0{\cdot}50^{a}$	$2{\cdot}92 \pm 0{\cdot}25^{a}$	$4{\cdot}34 \pm 0{\cdot}50^{a}$
	T7–12	$2{\cdot}20 \pm 0{\cdot}81^{b}$	$3{\cdot}80 \pm 1{\cdot}55^{ab}$	$4{\cdot}87 \pm 0{\cdot}80^{a}$
	T13–18	$3{\cdot}44 \pm 0{\cdot}52^{a}$	$4{\cdot}34 \pm 0{\cdot}32^{b}$	$4{\cdot}63 \pm 0{\cdot}14^{a}$

* Figures with the same letter are not significantly different from each other for a particular agar type and sampling time ($\alpha = 0{\cdot}05$)

correlation could be found between the numbers of bacteria present in the culture water and those associated with *Artemia*, except for the proportional increase in *Vibrio* spp. and *Pseudomonas* spp. with sampling time in both cases.

r- and K-strategists

By means of statistical analysis, significant differences in the proportion of *r*-strategists in the culture water have been found with sampling time and among the different tank series (Table 3). Table 3 reveals a general increase in proportion of *r*-strategists present in the culture water with sampling time. After 36 h, significant differences in the proportion of *r*-strategists occurred among the different tank series. The microbial communities of the third series already consisted mainly of *r*-strategists (78·7%) from the first sampling time, while the tanks of the first series contained only a low proportion of *r*-strategists at this sampling time (18·5%). The tanks of the second series occupied an intermediate position. At later incubation times, the microbial community was largely dominated by *r*-strategists in all the tanks (± 90%).

Table 3 The percentage of *r*-strategists present in the culture water of *Artemia* at the different sampling times and for the three tank series (mean ± S.D., $n = 6$)

	Sampling time (h)		
	36	84	132
T1–6	$18{\cdot}5 \pm 6{\cdot}6^{a*}$	$90{\cdot}0 \pm 7{\cdot}5^{cd}$	$94{\cdot}0 \pm 5{\cdot}4^{cd}$
T7–12	$58{\cdot}5 \pm 17{\cdot}5^{b}$	$90{\cdot}8 \pm 4{\cdot}9^{cd}$	$93{\cdot}7 \pm 6{\cdot}3^{d}$
T13–18	$78{\cdot}7 \pm 19{\cdot}5^{c}$	$91{\cdot}8 \pm 7{\cdot}5^{cd}$	$87{\cdot}5 \pm 16{\cdot}9^{cd}$

* Figures with the same letter are not significantly different from each other ($\alpha = 0{\cdot}05$).

The data on the proportion of *r*-strategists among the gut- and surface-associated bacteria ranged from $60{\cdot}8 \pm 32{\cdot}6$ to $90{\cdot}8 \pm 8{\cdot}9$% (data not shown). Statistical analysis of those data showed no relevant significant differences, neither with sampling time nor tank series. These data reveal the presence of a high proportion of *r*-strategists among the bacteria associated with *Artemia*, independently of the sampling time or tank series.

Microbial community analysis using Biolog

Functional diversity. Functional diversity was quantified at each sampling and incubation time for all 18 tanks. The substrate equitability ($\mathcal{J}$) ranged from $0{\cdot}916 \pm 0{\cdot}056$ to $0{\cdot}976 \pm 0.025$ and no clear trends or significant differences were found. Therefore, it can be concluded that the functional diversity of the microbial community was high under all the circumstances of the experiment.

Multivariate analysis. The results of the PCA performed separately for each sampling time on the Biolog profiles of the 18 tanks are shown in Fig. 1. The declared cumulative variance based on the first three principal components in Fig. 1 ranged from 61·7 to 65%. Although starting and operating conditions were supposed to be identical from the zootechnical point of view, three important groups are clearly separated after 12 and 36 h according to the series to which the culture tanks belonged, i.e. one large group contains tanks 13–18 (the third series), a second group tanks 7–12 (the second series) and a third group, tanks 1–6 (the first series). This means that consistent differences occurred among the microbial communities developing in the tanks of each series at the beginning of the culture period. At 84 and 132 h, scattering in the three series seemed to occur. Nevertheless, even at those later sampling times, the microbial communities of the six tanks of the third series remained relatively related to each other. This is better illustrated in Fig. 2 showing the rescaled dendrogram of the tanks at the end of the culture period (132 h). After 132 h, the characteristics of the 18 tanks have evolved in such a way that the clusters are no longer well related to the start-up series, except for tanks 13–18 (the third series) which remained well together.

Pearson correlation coefficient. To assess the effective differences between two Biolog profiles, the Pearson correlation coefficient as a similarity index was calculated. Subsequently, the means and standard deviation of the Pearson correlation coefficients were determined among and within the Biolog

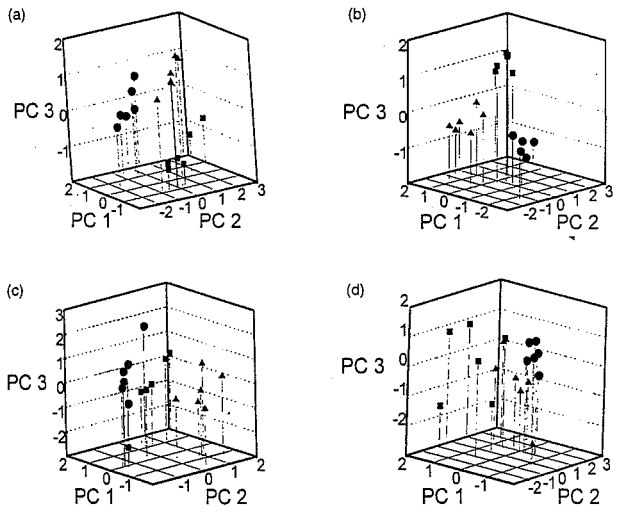

Fig. 1 Results of the PCA on the Biolog profiles of the 18 *Artemia* culture waters at the different sampling times (a) 12 h, (b) 36 h, (c) 84 h, (d) 132 h, and after 48 h of incubation. ▲, Series 1 (T1–6); ■, series 2 (T7–12); ●, series 3 (T13–18)

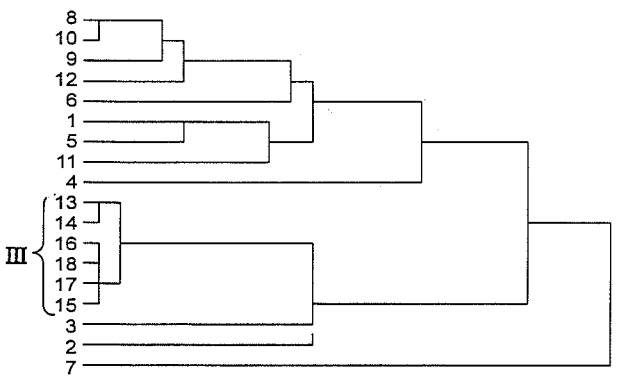

Fig. 2 The rescaled dendrogram of the Biolog profiles of the 18 *Artemia* culture waters after 132 h (T1–6, series 1; T7–12, series 2; III, the tanks of series 3, i.e. T13–18)

profiles of the tanks of the different series (Table 4). Rather than relative differences expressed in the rescaled dendrograms (Fig. 2), the Pearson correlation coefficient reflects the effective differences among and within the series of tanks. Generally, the Pearson correlation coefficients were high (>0·75) (Table 4). The mean Pearson correlation coefficient within the series was also generally lower than between two series after 12 and 36 h. After 132 h, the mean Pearson correlation coefficient within a series was generally lower than at the earlier sampling times, showing that the differences between the different tanks of one series are then of the same order of magnitude as those within the tanks of two different series. The fact that the tanks of the third series remained quite close to each other in terms of Pearson correlation coefficient as compare to the other values, confirms the observations of Figs 1 and 2.

Zootechnical results

From the animal densities and the length measurements, the rearing success of the *Artemia* culture could be evaluated and expressed as percentage survival and biomass concentration (Table 5). Based on the author's own experience, a hypothetical ideal case is also given, assuming 75% survival after

Table 4 Similarity matrix of the Biolog profiles of the *Artemia* culture waters with the Pearson correlation coefficient within and among the three series of six tanks (mean ± S.D.)

	T1–6	T7–12	T13–18
Sampling after 12 h			
T1–6	0·983 ± 0·007	—	—
T7–12	0·950 ± 0·012	0·978 ± 0·008	—
T13–18	0·932 ± 0·010	0·899 ± 0·020	0·982 ± 0·006
Sampling after 36 h			
T1–6	0·980 ± 0·008	—	—
T7–12	0·920 ± 0·023	0·961 ± 0·019	—
T13–18	0·952 ± 0·014	0·888 ± 0·049	0·974 ± 0·011
Sampling after 84 h			
T1–6	0·953 ± 0·016	—	—
T7–12	0·903 ± 0·024	0·892 ± 0·038	—
T13–18	0·927 ± 0·012	0·890 ± 0·033	0·952 ± 0·013
Sampling after 132 h			
T1–6	0·886 ± 0·046	—	—
T7–12	0·879 ± 0·044	0·906 ± 0·048	—
T13–18	0·866 ± 0·045	0·834 ± 0·066	0·936 ± 0·048

7 d and a length of 2·75 mm. Statistical analysis revealed significant differences in rearing success between the tank series. In terms of biomass production, there were already significant differences after 12 h as the tanks of series one yielded lower biomass concentration than the tanks of series two and three, indicating that the differences appear from the onset of the culture. Only the rearing success of the tanks of series three meets the ideal both in terms of biomass production and survival.

DISCUSSION

Thirty-six hours after the feeding of the *Artemia* has started, the counts on marine agar increased from 10^3–10^4 to 10^8–10^9 cfu ml^{-1} (Table 1). Later on, a decrease to 10^6–10^7 cfu ml^{-1} was observed. Rosowski *et al.* (1992) counted on plate count agar approximately the same maximal bacterial density in *Artemia* culture water at 25°C. A similar bacterial development up to 10^7 cfu ml^{-1} from hatching until 48 h later has been reported by Austin and Allen (1981). A similarly rapid increase from 10^3 to 10^6 cfu ml^{-1} due to enrichment of the environment was also observed in the hatching of cod (*Gadus morhua*) and halibut (*Hippoglossus hippoglossus*) larvae (Hansen and Olafsen 1989). Generally, bacterial loads reflect the nutrient richness of the environment (De Leij *et al.* 1993), which must explain these observations. The sharp decrease at later sampling times observed in this study indicates a stabilization of the culturable bacterial numbers at around 10^6 cfu ml^{-1}, probably due to grazing by *Artemia*. *Artemia* are, in fact, non-selective filter-feeders, ingesting all kinds of particles to a maximum size of 25–30 μm for nauplii to 40–50 μm for adults (Dobbeleir *et al.* 1980). Rosowski *et al.* (1992) did not notice such a sharp decrease in bacterial densities on plate count agar during this time span, probably as a consequence of lower animal densities (2·5–3·0 ml).

Environmental conditions select organisms that either multiply/grow rapidly under uncrowded, nutrient-rich conditions (*r*-strategists), or that can efficiently exploit resources under crowded conditions (*K*-strategists). In general, *r*-strategists are characteristic for unstable environments, while *K*-strategists do better in stable environments (Pianka 1970). According to the definitions of Andrews and Harris (1986), the intensive *Artemia* juvenile culture shows characteristics of an uncrowded bacterial environment, with low initial bacterial densities due to the chlorination of the sea water and apparently negligible density-dependent growth factors. These observations appear to be confirmed by this study, as after 84 h the microbial community was, in all cases, dominated by *r*-strategists (± 90%, Table 3). Significant differences in the proportion of *r*-strategists occurred only at the first sampling time. As environmental conditions were identical, these differences must be due to differences in the bacterial inoculum originally present.

In an attempt to manipulate the bacterial community dur-

Table 5 The rearing success of the *Artemia* culture for the three tank series expressed in biomass concentration (mg dry weight l^{-1}) and survival (%) (mean ± S.D.)

		Sampling time (h)		
		12	84	132
Ideal case	biomass	25	73·8	165
T1–6	biomass	18·0 ± 0·8^{a*}	32·9 ± 5·2^{a}	–
	survival		69·6 ± 5·0^{1}	50·3 ± 8·5^{1}
T7–12	biomass	23·9 ± 1·8^{b}	55·3 ± 9·9^{b}	72·4 ± 31·4^{a}
	survival		80·5 ± 8·4^{12}	50·6 ± 15·1^{1}
T13–18	biomass	24·1 ± 2·8^{b}	74·1 ± 13·9^{c}	152·5 ± 27·3^{b}
	survival		85·8 ± 13·7^{2}	79·9 ± 11·8^{2}

*Numbers with different letters (for biomass) and figures (for survival) are significantly different from each other for a particular sampling time; –, no results available.

ing the hatching of turbot (*Scophthalmus maximus*), Salvesen *et al.* (1995) counted, in tanks with so-called microbially-saturated water, a 2-fold higher proportion of *K*-strategists than in those receiving filtered sea water. At the end of the stagnant period (day 5), this proportion was high in all tanks. The increase in the fraction of *K*-strategists contrasts with the increase in the fraction of *r*-strategists observed in this study (Table 3). This may be attributed to the much higher input of organic matter (feed) in the culture water of *Artemia* compared to that of the turbot larvae, as the turbot larvae were fed with rinsed live prey. In the case of the *Artemia* culture, the environment has a much higher carrying capacity due to the high nutrient input, resulting in fast development of the bacteria with the highest growth rate, i.e. *r*-strategists.

In the case of the surface- and gut-associated bacteria, the percentage of *r*-strategists was relatively high (60–90%) during the whole experiment, and no significant differences could be found among the sampling times or tank series. This may be explained by the fact that surface growth is subject to strong *r*-selection, as *Artemia* moult more or less daily instead of growing continuously (Sorgeloos *et al.* 1986).

Although there is evidence that some strains are unable to contribute to profile formation in Biolog GN microtitre plates (Haack *et al.* 1995; Heuer *et al.* 1995), this system seems to be a powerful tool to discriminate between the bacterial communities occurring in the intensive culture of *Artemia* juveniles. Clearly distinct Biolog profiles could be attributed to the tanks of the different series or to the different sampling times, and could be discriminated through PCA and CA.

This study reveals that uncontrolled factors have an important impact on the establishment and evolution of the microbial community in the culture tanks; an obvious clustering of the Biolog fingerprints appeared and remained in series three throughout the development of the *Artemia* culture (Figs 1 and 2). Nevertheless, even if the PCA and CA revealed significant differences in metabolic fingerprint (Figs 1 and 2), the similarities between the microbial communities as determined by the Pearson correlation coefficient were high in all cases (Table 4). This suggests that the metabolic abilities of the microbial communities which developed in the culture tanks were not only the result of deterministic factors, but also of stochastic factors (Moriarty and Body 1995). Deterministic factors governing the microbial communities, such as the salinity and the temperature of the culture water and feed concentration, were set to be identical in the three series of tanks, which may explain why the Pearson correlation coefficients between the fingerprints were high (Table 4). However, stochastic phenomena may be responsible for the differences observed with PCA and CA; different microbial species can enter the different tanks and chance favours those organisms which happen to be in the right place at the right time to respond to the sudden increase in nutrients originating from the *Artemia* feed. As a consequence, the microbial community which develops in the culture tank is influenced both by the environmental conditions and chance. This would suggest that the variability of the microbial community associated with the culture of *Artemia* juveniles can be restricted through the early application of a probiotic strain or a mixture of bacteria, provided they are well adapted to the prevailing conditions.

The variation observed among the different series of tanks must be due to differences in inoculum at the beginning of the culture, because clearly distinct microbial communities had already appeared after 12 h. Chlorination of the sea water prior to the culture did not disinfect it efficiently, as a considerable amount of bacteria remained (10^3–10^4 cfu ml^{-1}). Also, the feed seemed to be a major source of bacteria ($\pm 10^3$ cfu ml^{-1} d^{-1}); conversely, aeration made a negligible quantitative contribution ($\pm 0{\cdot}05$ cfu ml^{-1} d^{-1}). As the cysts originated from the same batch, they could not explain the observed differences in microbial community. As the feed also came from the same batch, it is very likely that the sea water was responsible for the differences in microbial community, as that used for the different series was taken from the North Sea at different times. It has been observed in *Artemia* culture using filter-sterilized sea water that the large numbers of bacteria in hatching water resulted from a gradual quantitative increase in most of the taxa originally present in the cysts (Austin and Allen 1981). This confirms that the inoculum originally present has a determining influence on the microbial community which will develop, and that this influence may last several days, even in a very *r*-selective environment. This can be seen in the 3D-plots of the principal components (Fig. 1) and the dendrogram (Fig. 2). The variability observed among the tanks of the same series might be explained by small differences in the inoculum of each tank. Even after chlorination, different types of bacteria still occur on the surface and in small fissures of the walls of the culture tanks (data not shown). For instance, when the walls of four different culture tanks were sampled after disinfection and plated onto marine agar, 19 morphologically different colony types were detected. Of these 19 culturable colony types, 13 were present in only one of the four culture tanks, five in two of the four culture tanks, one in three of the four tanks and not one single morphological type was found to be present in all of the sampled culture tanks. This heterogeneity might give rise to qualitative and quantitative differences in the inocula. Furthermore, aeration causes turbulences above the culture tanks which may lead to an additional inoculation with bacteria from the ambient air of the rearing room. Repetitive sampling of the ambient air at different places in the rearing room revealed significant differences in microbial communities both in space and time (data not shown). In three places, the air was sampled twice with a one month interval. The plate counts on marine agar ranged from 124 to 5345 cfu m^{-3}. These densities are in the

normal range (Verstraete and Voets 1974), but indicate major differences in the indoor micro-climates. The morphological colony types present on marine agar also differed drastically, even within the samples taken at the same place but at different times. These stochastic phenomena may be at the origin of the differences occurring among the tanks of the same series.

A striking observation in Fig. 1 and 2 is that the bacterial communities of the tanks of the third series remain better clustered throughout the experiments, compared with the tanks of the two other series. In Table 4, it can be seen that at later sampling times, the mean Pearson correlation coefficient among the tanks of series three is higher (0·936) than among those of series one (0·886) and series two (0·906). Table 3 shows that the tanks of the third series also have the highest proportion of *r*-strategists after 36 h. It seems that the inoculum of the third series is, from the start, well adapted to the conditions occurring in the *Artemia* culture, and that these microbial communities undergo the least changes in time.

It is interesting to observe the parallelism between the zootechnical results and the Biolog profiles of the corresponding cultures. Differences in Biolog profiles, at least at the earlier sampling times (Fig. 1), coincide with the differences in rearing success among the three tank series (Table 5). The best zootechnical results were obtained in the tanks of the series with the highest proportion of *r*-strategists (Table 3) and the highest bacterial counts on TCBS and, to a lesser extent, *Pseudomonas* agar (Table 1). These observations suggest that a correlation exists between the characteristics of the bacterial community present and the zootechnical performance of the *Artemia* culture. Some bacteria are more desirable than others in the culture of *Artemia*. Several pathogens have been reported in the literature (Solangi *et al.* 1979; Austin and Allen 1981), especially *Vibrio* spp. (Gunther and Catena 1980; Puente *et al.* 1992; Rico-mora and Voltolina, 1995). However, in this study the best zootechnical results were obtained in the tanks showing the highest densities of *Vibrio* spp. (Table 1), indicating the ambiguity of these bacteria with regard to the effects on cultured species. However, bacteria have also been reported to have a beneficial effect or even to be essential in the culture of *Artemia* (Yasuda and Taga, 1980; Intriago and Jones, 1993; Rico-mora and Voltolina 1995; Gorospe and Nakamura 1996). It is assumed that this correlation is causal and experiments are being set up to evaluate this further.

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